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(54) Title: <b>ANTI-EGF-R/ANTI-CD3 BISPECIFIC MONOCLONAL ANTIBODY, METHOD FOR ITS PRODUCTION AND ITS USE</b>			
(57) Abstract			
<p>It is described an anti-EGF-R/anti-CD3 bispecific monoclonal antibody (bimAb) of hybrid isotype (IgG1/IgG2a). It is also described the construction of a hybrid hybridoma secreting such bimAb, and the purification of anti-EGF-R/anti-CD3 bimAb molecules from hybrid hybridoma performed by protein-A cation exchange chromatography. Said bimAb turned out to be useful against tumor cells showing the epidermal growth factor receptor (EGF-R+).</p>			

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Anti-EGF-R/anti-CD3 bispecific monoclonal antibody, method for its production and its use.

Field of the invention

The present invention relates to the construction of a hybrid hybridoma secreting a new anti-EGF-R/anti-CD3 bispecific monoclonal antibody 5 (bimAb) of hybrid isotype (IgG1/IgG2a) useful in tumor therapy of cells showing the epidermal growth factor receptor (EGF-R+).

Further, the present invention relates to such antibody and to its purification thereto. Such purified antibody was able to trigger the lysis of EGF-R+ tumor cell lines (for example A431, IGROV-1, MDA-468 10 and U-87) and of NIH-3T3 transfected cells expressing the human EGF-R, by cytolytic T lymphocytes, but it was ineffective on EGF-R- tumor targets (where the signs + and - associated to EGF-R indicate respectively the presence or the absence of said receptor on cells).

State of the art

15 Anti-tumor/anti-CD3 bispecific monoclonal antibodies are useful reagents able to confer anti-tumor specificity to T lymphocytes.

Ferrini et al., 1993, Cancer Detection and Prevention, 17(2), 295-300, using two different approaches constructed an anti-EGF-R/anti-CD3 bimAb, with the aim of specifically targeting T lymphocytes against tumor cells 20 expressing the epidermal growth factor receptor (EGF-R+). In the first case, they constructed, by somatic fusion, a hybrid hybridoma secreting said anti-EGF-R/anti-CD3 bispecific monoclonal antibody (bimAb). In the second case, the bimAb was prepared by chemical linking. The *in vitro* functional activity of these two bimAbs indicated that the bimAb secreted 25 by the hybrid hybridoma was more efficient to induce the cytolysis of EGF-R+ tumor cells by CD8+ T cell clones than the chemically prepared

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bimAb.

The authors of the present invention constructed a hybrid hybridoma secreting an anti-EGF-R/anti-CD3 bispecific monoclonal antibody (bimAb) and they compared such antibody to the above mentioned one (kindly provided by Dr.Ferrini and hereinafter named "Ferrini bimAb") which, as far as the applicant knows, represents the only anti-EGF-R/anti-CD3 bimAb which has ever been disclosed.

Summary of the invention

The authors of the present invention have surprisingly found that the bimAb prepared according to the present invention (obtained from hybrid 10 hybridoma deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH: DSM ACC2151) shows a different behaviour and a cytolytic activity ten times higher than the known bimAb (Ferrini bimAb). A fundamental characteristic of the present invention therefore refers to 15 a hybrid hybridoma deposited at DSM (DSM ACC2151) and to the bispecific monoclonal antibody secreted by such hybrid hybridoma, and that is the anti-EGF-R/anti-CD3 bimAb of hybrid isotype (IgG1/IgG2a) hereinafter named DSM ACC2151 bimAb.

A further aspect of the present invention refers to the preparation of 20 DSM ACC2151 hybrid hybridoma and to the recovery of bimAb secreted by said hybridoma.

A further characteristic of the present invention refers to the purification of such DSM ACC2151 bimAb by a Protein-A chromatography followed by a cation exchange chromatography.

25 The present invention also refers to anti-EGF-R (DSM ACC2150) and anti-CD3 (DSM ACC2152) hybridomas used for the preparation of DSM ACC2151 hybrid hybridoma, to monoclonal antibodies produced by such hybridoma and to the method for their production.

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The present invention further refers to the production of a pharmaceutical composition comprising a monoclonal antibody or its fragments, for example  $F(ab')_2$  fragment, according to the invention for the treatment of tumors, optionally in combination with a 5 pharmaceutically acceptable carrier and/or excipient.

Reference to drawings and to sequence lists

- Figure 1 shows the effect of DSM ACC2151 hybrid hybridoma supernatant on the cytolytic activity of CD3+8+ BC1 cytolytic clone against the EGF-R+ A431 target cell line in a 4-hour  $^{51}\text{Cr}$ -release test.
- 10 The abscissa reports the E:T ratio (E = effector cell; T = target cell). The ordinate reports the % of lysis of the target cells.
- Figure 2 shows the effect of different concentrations of the purified DSM ACC2151 bimAb on the cytolytic activity of a cytotoxic CD8+ polyclonal cell line against EGF-R+ normal and neoplastic target cells.
- 15 The abscissa reports in logarithmic scale the bimAb concentration (ng/ml). The ordinate reports the % of lysis concentration of the target cells.
- Figure 3 shows the effect of DSM ACC2151 bimAb on the cytolytic activity of different populations of effector cells against the EGF-R+ A431 tumor cell line. The abscissa reports the E:T ratio of effector cells/target cells. The ordinate reports the % of lysis of target cells.
- 20 - Figure 4 shows the effect of DSM ACC2151 bimAb and DSM ACC2152 mAb on the intracellular free  $\text{Ca}^{++}$  concentration on CD8+ polyclonal cell lines. In panel A, a rabbit anti-mouse Ig antibody was added as negative control. In panels B and C, DSM ACC2152 mAb and DSM ACC2151 bimAb were respectively used, followed by the addition of anti-mouse Ig antiserum as cross-linking agent. In panel D, cells were coated with DSM ACC2151 bimAb and cultured for 24 hours in the absence of soluble DSM ACC2151 bimAb.

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Before the experiment an anti-mouse IgG was added to the test. The ordinate reports the  $\text{Ca}^{++}$  concentration in nM.

- Figure 5 shows the capacity of effector cells coated with DSM ACC2151 bimAb to retain the ability to lyse EGF-R<sup>+</sup> target cells for a prolonged

5 time.  
A CD8+ polyclonal cell line was coated with DSM ACC2151 bimAb (100 ng/ml for 1 hour at 4°C) and cultured for 24 hours in absence of soluble DSM ACC2151. Data are expressed as % of  $^{51}\text{Cr}$ -release from A431 target cells at a 10:1 effector/target cell ratio.

10 - Figure 6 shows analytical MonoS chromatography (Pharmacia) of material eluted from Protein A chromatography of DSM ACC2151 hybrid hybridoma conditioned medium.

- Figure 7 shows analytical MonoS chromatography (Pharmacia) of DSM ACC2151 bimAb purified by cation exchange chromatography on S-Sepharose FF (Pharmacia).

15 - Figure 8 shows an electrophoretic analysis by sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) gel of purified fractions of DSM ACC2151 bimAb.

- Figure 9 shows the cytolytic activity of DSM ACC2151 bimAb if compared to that of Ferrini bimAb. The abscissa reports, in logarithmic scale, the amounts expressed in ng/ml. The ordinate reports the percentage of lysis according to an effector cells (lymphocytes)/target cells (tumor cells) ratio of 5/1.

20 - SEQ ID NO:1 reports the oligonucleotide sequence of the heavy chain variable (VH) region of DSM ACC2152 mAb.

25 - SEQ ID NO:2 reports the amino acid sequence of VH region of DSM ACC2152 mAb.

- SEQ ID NO:3 reports the oligonucleotide sequence of the light chain

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variable (VL) region of DSM ACC2152 mAb.

- SEQ ID NO:4 reports the amino acid sequence of VL region of DSM ACC2152 mAb.

- SEQ ID NO:5 reports the nucleotide sequence of the primer named VH1BACK 5 HindIII for use in PCR. - SEQ ID NO:6 reports the nucleotide sequence of the primer named VHFOR-LINKER SCA for use in PCR. - SEQ ID NO:7 reports the nucleotide sequence of the primer named VLBACK-LINKER SCA for use in PCR. - SEQ ID NO:8 reports a nucleotide sequence of the primer named VLFOR UNIVERSAL EcoR1 for use in PCR.

10 Detailed description of the invention

The anti-EGF-R/anti-CD3 bispecific monoclonal antibody of IgG1/IgG2a hybrid isotype is secreted by a hybrid hybridoma obtainable by somatic hybridization of a hybridoma secreting an anti-EGF-R monoclonal antibody and a hybridoma secreting an anti-CD3 monoclonal antibody.

15 The method for the production of a hybrid hybridoma secreting an anti-EGF-R/anti-CD3 bispecific monoclonal antibody of IgG1/IgG2a hybrid isotype according to the invention therefore comprises the somatic hybridization of a hybridoma secreting an anti-EGF-R monoclonal antibody and a hybridoma secreting an anti-CD3 monoclonal antibody.

20 Further, the method for the production of an anti-EGF-R/anti-CD3 bispecific monoclonal antibody according to the invention comprises the somatic hybridization of a hybridoma secreting an anti-CD3 mAb and a hybridoma secreting an anti-EGF-R mAb, and then the purification of such antibody (preferably, first by Protein-A chromatography and then by 25 cation exchange chromatography).

The bimAb according to the invention can be easily and efficiently purified from parental mAbs (i.e. anti-CD3 mAb and anti-EGF-R mAb) by Protein-A chromatography followed by cation exchange chromatography.

In any citation here made, the hybridoma secreting the anti-EGF-R mAb is preferably the hybridoma named Mint5 (described in patent application PCT/EP94/02969 of the same applicant) and deposited as DSM ACC2150, while the hybridoma secreting the anti-CD3 mAb is preferably the hybridoma 5 deposited as DSM ACC2152.

The bispecific antibody according to the invention can be obtained also in form of recombinant single chain by genic fusion of sequences of variable regions of the light and heavy chain of genes encoding an anti-CD3 monoclonal antibody and an anti-EGF-R monoclonal antibody. 10 respectively, according to the known techniques. Anti-CD3 mAb is preferably secreted by DSM ACC2152 hybridoma, while anti-EGF-R mAb is preferably secreted by DSM ACC2150 hybridoma.

The purified bimAb according to the invention was able to trigger the lysis of EGF-R+ tumor cell lines (for example A431, IGROV-1, MDA468, U-15 87, etc.) and of NIH-3T3 transfectants expressing human EGF-R by cytolytic T lymphocytes, but it was ineffective to stimulate the lysis of EGF-R- tumor targets. Normal EGF-R+ cells (keratinocytes and endometrial cells) were also susceptible to cytolysis only at saturating amounts of bimAb (Fig.2).

20 The ability of the bimAb according to the invention to deliver activation signals to T cells was evaluated by Ca<sup>++</sup> mobilization and lymphokine production experiments. The bimAb according to the invention, when administered in *in vitro* studies in soluble form, failed to induce an intracellular Ca<sup>++</sup> increase, which occurred only after cross-linking 25 induced by an anti-mouse IgG antibody (Fig.4). Secretion of lymphokines (IFN-gamma, TNF- $\alpha$  and GM-CSF) was induced only by contact of the bimAb coated effector cells with the specific tumor target, according to the invention.

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The data hereinafter reported indicate that the bimAb according to the invention induce efficiently and specifically the secretion of lymphokines from T lymphocytes by contact with EGF-R+ tumor cells. In addition, it was observed that the effector cells coated with the bimAb according to the invention retained the ability to recognize and to lyse EGF-R+ tumor cells for a prolonged period (up to 24 hours).

These data indicate that the activation of effector cells by the bimAb according to the invention can only occur at the tumor site, where cross-linking of surface CD3 molecules is induced by contact with tumor cells.

On the basis of these results, it appears that T lymphocytes coated with the bimAb according to the invention could be used, for example, for: a) local treatment of tumors expressing EGF-R molecules, provided that tissues surrounding the tumor are EGF-R-; or b) systemic treatment of tumors overexpressing EGF-R. With regard to the therapeutic possibilities, recent studies suggest a clinical efficacy of immunotherapy with lymphocytes coated with anti-NCAM/anti-CD3 bimAb in the treatment of gliomas (Nitta et al., 1990, Lancet, 335, 368-376).

It was eventually studied the activity of DSM ACC2151 bimAb compared to that of the known bimAb (i.e. Ferrini bimAb) and it was observed that DSM ACC2151 bimAb was about 10 times more active than Ferrini bimAb (Fig.9).

The realization of the present invention will result clearer by way of the following Examples.

Example 1

Production of anti-CD3 monoclonal antibody (mAb) (DSM ACC2152)

25 Isolation and culture of T lymphocytes

Peripheral blood mononuclear cells (PBMNC) were isolated from 20 ml of heparinized peripheral blood by centrifugation on Ficoll Hypaque<sup>R</sup> density gradient. After three washes in RPMI 1640 medium (Seromed, Berlin,

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Germany), supplemented with 10% heat-inactivated FCS (Fetal calf serum, PAA, Austria), penicillin-streptomycin (1% vol/vol of a commercial solution; Seromed, Berlin, Germany) and 200 mM L-Glutamin (complete medium) cells were resuspended in the same medium and brought to the 5 concentration of  $10^6$ /ml.

T lymphoblasts for immunization were produced by culture for 3 days in complete medium with 1% vol/vol PHA (Phytohemagglutinin, Gibco, NY, USA) and cultured 3 days in 24 well Costar<sup>R</sup> plates. Lymphoblasts were then expanded by culture in complete medium supplemented with 50 UI/ml of 10 recombinant IL2 (Cetus Corporation, CA, USA).

Immunization fusion and screening

Six-week-old Balb/c female mice were immunized by four weekly intraperitoneal injections of  $10^7$  cultured lymphoblasts obtained by culture as in the previous step. T lymphoblasts from different cultures 15 were pooled together and washed two times in PBS and resuspended in 0.2 ml PBS for injection. After 10 days mice received a booster injection of  $10^7$  cells, followed by splenectomy 3 days later. The spleen was disaggregated by the use of a siringe and a 22 gauge needle.  $10^8$  immune 20 splenocytes were washed 3 times in RPMI 1640 medium without FCS and pelleted together with  $10^7$  P3U1 myeloma cells. The fusion was performed by dropwise addition of 0.5 ml of 40% polyethylenglycole (Merk) in RPMI 25 1640 at 37°C. After 5 minutes, 25 ml of RPMI 1640 medium without FCS were added slowly to the fusion at 37°C followed by 25 ml of RPMI 1640 with 10% FCS. Cells were centrifuged, resuspended in complete RPMI 1640 medium and plated in 24 well Costar<sup>R</sup> plates containing Balb/c peritoneal macrophages as feeder layer. After 24 hours of culture HAT (Hypoxantine Aminopterine Thymidine) was added to the cultures (2% of a 50x stock 30 solution, Flow Labs, UK). Medium with HAT was replaced every day. After

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10-15 days wells were scored for proliferation. Supernatants were screened for their ability to induce the proliferation of PBMNC in a 3 days proliferation assay. To this end  $10^5$  PBMNC cells from healthy donors were plated in triplicate wells in U-bottomed 96 well microtiter plates 5 in the presence of 20  $\mu$ l of hybridoma supernatant or 1% PHA (as positive control). After 3 days cultures were pulsed with 0.5  $\mu$ Ci of methyl- $^3$ H-thymidine (specific activity 5 Ci/mMol; Amersham, UK) for additional 18 hours. Cultures were then harvested onto glass fiber filters by a cell harvester and counted for  $^3$ H-thymidine uptake in a  $\beta$ -counter. The 10 MRSF43.1.16 hybridoma of IgG2 isotype, deposited as DSM ACC2152, producing significant proliferation was subcloned four times by limiting dilution.

Immunofluorescence analysis

The reactivity of DSM ACC2152 mAb was compared to that of OKT3 (anti-CD3; 15 Ortho, NJ, USA) on a panel of different cells by indirect immunofluorescence and cytofluorimetric analysis. A pellet of  $5 \times 10^6$  cells was incubated at 4°C for 30 minutes with 50  $\mu$ l of hybridoma supernatant. After on washing with PBS, said cells were incubated for additional 30 minutes by adding a fluorescein isothiocyanate (FITC)-conjugated goat 20 anti-mouse immunoglobulin (Ig) antibody (second antibody). As negative control, cells were incubated with second antibody only. Data obtained on a panel of normal and neoplastic cells of different histotypes cells are summarized in the Table 1 below reported. These data demonstrated an identical pattern of reactivity of OKT3 and DSM ACC2152 mAb, referred to 25 the identification of the CD3 molecule expressed on mature cells.

Table 1

Cells/histotype	OKT3	DSM	ACC2152
PBMNC (3x)	65%	64%	
Cultured T lymphoblasts	98%	99%	
CD4+ T cell clones (3)	99%	99%	
CD8+ T cell clones (4)	98%	100%	0%
CD16+ NK clones (3)	0%		
Jurkat/T cell leukemia	99%	98%	
HPB-All/T cell leukemia	96%	95%	1%
IM9/B cell leukemia	0%	0%	
Daudi, Raji/Burkitt lymphoma	1%	1%	
K562, HL-60/myeloid leukemia	0%	0%	
IGROV1, SW626/ovarian cancer	0%	0%	
MCF-7/ breast carcinoma	1%	0%	

Data are expressed as % of positive cells

Isolation, cloning and sequencing of heavy (H) and light (L) variable (V) regions from DSM ACC2152

mRNA from DSM ACC2152 hybridoma cells was extracted by the use of "Total RNA Separator" kit (Clontech Laboratories Inc., CA. 94303, USA). Heavy chain (VH) and light chain (VL) variable region cDNAs were obtained by reverse transcriptase reaction and were PCR amplified (GeneAmp RNA PCR Kit-Perkin Elmer Corporation, CT.06859, USA). The PCR oligoprimer used for PCR amplification were synthesized according to Orlandi et al., 1989.

10 PNAS, USA, 26, 3833-3837.

The amplified variable regions were cloned in the HindIII-EcoRI restriction sites of pUC18 plasmid for sequencing. To introduce HindIII and EcoRI restriction sites in the variable regions, they were reamplified and linked together according to Davis et al., 1991.

15 Bio/Technology 9, 165-169, using the following primers:

5' VH: VH1BACK HindIII: (SEQ ID NO:5)

3' VH: VHFOR-LINKER SCA: (SEQ ID NO:6)

5' VL: VLBACK-LINKER SCA: (SEQ ID NO:7)

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3'VL: VLFOR UNIVERSAL EcoR1: (SEQ ID NO:8)

Heavy and light chain variable regions were sequenced by Sanger method by the use of "Sequenase Version 2.0" kit (United States Biochemical Corporation, Ohio 44122, USA).

5 DSM ACC2152 mAb VH nucleotide is reported in SEQ ID NO:1.

DSM ACC2152 mAB VL nucleotide is reported in SEQ ID NO:3.

The DSM ACC2152 VH and VL DNA sequences were analyzed for homology against PCgene data base (Intelligentics 1992) and were confirmed as new sequences (being not present in the data bank).

10 Example 2

Production of bispecific monoclonal antibody (bimAb) DSM ACC2151

Cell fusion and screening of hybrid hybridomas secreting bimAbs

The Mint-5 (IgG1) anti-EGF-R hybridoma (described in the copending patent application PCT/EP94/02969 of the same applicant) was deposited as DSM

15 ACC2150.

DSM ACC2150 and DSM ACC2152 hybridomas were used for the preparation of a hybrid hybridoma named B/MRS 26.1.17 deposited as DSM ACC2151 secreting the bimAb of interest.

20 Hypoxantineguanine phosphoribosyltransferase (HGPRT)-deficient mutants of the DSM ACC2150 hybridoma were selected by culture in the presence of increasing amounts of 8-azaguanine (1-50 µg/ml) (Sigma, St.Louis, MO).

A HGPRT-negative clone of the DSM ACC2150 hybridoma was fused with iodoacetamide-inactivated hybridoma cells of the DSM ACC2152 hybridoma using a standard polyethylene glycol fusion protocol (Clark et al., 1987,

25 J. Nat. Cancer Inst., 79, 1393-1401). Hybrid hybridomas (quadromas) were selected by culture in HAT medium. Hybrid hybridomas were cloned by limiting dilution and tested for antibody production. The culture medium for hybrid hybridomas was RPMI 1640 supplemented with 10% FCS and 2mM L-

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glutamine. The hybrid hybridoma supernatants were directly screened for their ability to induce the CD8+ cytolytic T cell clone BC1 to lyse the EGF-R+ A431 tumor target cells (Merlino et al., 1984, Science, 224, 417-419). As shown in Figure 1, a 1:100 dilution of the supernatant of the 5 selected hybrid hybridomas, the DSM ACC2151 secreting the bimAb, efficiently induced lysis of the A431 target cells. Parental anti-CD3 anti-EGF-R mAbs used alone or in combination failed to induce A431 cell lysis.

Assay for cytolytic activity

10 PBL, T cell lines and clones were used as effector cells at effector/target cell ratio ranging from 40:1 to 0.6:1 in a 4h  $^{51}\text{Cr}$ -release assay. The following cell lines were used as targets: IGROV-1 (ovarian carcinoma), MeWo (melanoma), Raji (Burkitt's lymphoma), MDA-468 (breast cancer), A431 (epidermoid carcinoma), U-87 (glioma), NIH-3T3 and NIH-3T3 15 EGF-R+ transfectants (kindly provided by Dr. P. Di Fiore, NIH, Bethesda, MD; Di Fiore et al., 1987, Cell, 51, 1063-1070). In addition, normal keratinocytes and endometrial cells from primary or secondary cultures were also used as targets.  $5 \times 10^3$   $^{51}\text{Cr}$ -labelled target cells were added to various numbers of effector cells in 96-well microtiter plates. For the 20 evaluation of mAb triggered cytotoxicity, various dilutions of supernatants of the hybrid hybridoma culture or of affinity chromatography mAb fractions were added at the onset of the assay. The final culture volume was 200  $\mu\text{l}$ . After 4h of culture, 100  $\mu\text{l}$  of supernatant were collected from each well and counted in a gamma-counter 25 for the evaluation of  $^{51}\text{Cr}$ -release. The percentage of lysis was calculated as described in Ferrini et al., 1987, J. Immunol., 138, 1297-1302. To verify the target specificity, the bimAb DSM ACC2151 was tested for its ability to induce cytolysis of EGF-R+ and EGF-R- tumor target

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cells.

Table 2

Effect of DSM ACC2151 bimAb on the cytolytic activity of a cytotoxic CD3+8+ polyclonal cell line against different tumor target cells.

Target	No. of EGF-R sites/cell $\times 10^3$ <sup>a</sup>	% of lysis in the presence of <sup>b</sup>		
		No. mAb	bimAb (100ng/ml)	anti-CD3+ anti-EGFR mAb
<b>EGF-R+ cell lines:</b>				
MDA-468	1700	8	38	8
A431	1400	2	50	2
NIH-3T3 EGF-R+	100	1	56	2
U-87	67	1	40	1
IGROV-1	50	10	71	10
<b>EGF-R- cell lines:</b>				
MeWo	- <sup>c</sup>	1	1	2
Raji	-	2	2	2
NIH-3T3	-	1	1	1

<sup>a</sup> determined by Scatchard analysis using  $^{125}\text{I}$ -mEGF (methionine-EGF)

<sup>b</sup> determined by a standard  $^{51}\text{Cr}$  release assay at a 10:1 effector/target cell ratio

<sup>c</sup> under the level of detectability

- 5 As shown in Table 2, the bimAm (100ng/ml) induced CD3+8+ cell lines to lyse EGF-R+ tumor cells, including A431, IGROV-1, MDA 468 and U-87. On the contrary the EGF-R- Raji, and MeWo tumor cell lines were not susceptible to lysis. Stable transfectants of the NIH3T3 cell line expressing human EGF-R were also efficiently lysed in the presence of 10 bimAb. Untransfected cells were not lysed indicating that the targeting

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activity of the bimAb is specifically dependent on the expression of the EGF-R molecule on target cells. It is noteworthy that the susceptibility to cytolysis appears to be dependent on the density of EGF-R molecules expressed with the exception of saturating amounts of bimAb (100 ng/ml).

5 However, dose/response experiments showed that, in the presence of low concentrations of bimAb, IGROV1 target cells were lysed considerably less efficiently than A431 target cells (Figure 2). In addition, the concentration of bimAb required to induce half-maximal cytolysis (ED<sub>50</sub>) were remarkably lower when A431 were used as target cells (ED<sub>50</sub> = 1.5 10 ng/ml) as compared to IGROV-1 (ED<sub>50</sub> = 8 ng/ml). Analysis of both EGF-R<sup>+</sup> normal cells (keratinocytes and endothelial cells) required higher amounts of bimAb for half-maximal cytolysis being ED<sub>50</sub> = 12 and 100 ng/ml, respectively, as compared with A431 cells (Figure 2). As expected, EGF-R<sup>-</sup> PHA-induced lymphoblasts were not lysed in the presence of DSM

15 ACC2151 bimAb.

Different populations of CD3<sup>+</sup> effector cells were tested for their ability to lyse EGF-R<sup>+</sup> target cells in the presence of DSM ACC2151 bimAb. CD4<sup>+</sup>, CD8<sup>+</sup> or TCRgamma/δ<sup>+</sup> T lymphocytes were mitogen-activated and expanded for 2-3 weeks in IL-2 containing medium. It was observed that 20 lysis of A431 target cells could be induced by PHA-activated IL-2 expanded T lymphoblasts but not by resting PBL. Both CD8<sup>+</sup> and TCRgamma/δ<sup>+</sup> cells efficiently lysed A431 in the presence of DSM ACC2151 bimAb, while CD4<sup>+</sup> cells were poorly effective (Figure 3).

Similar results were obtained using PBL and purified populations isolated 25 from three additional healthy donors. It should be noted, however, that the level of cytolytic activity induced by DSM ACC2151 bimAb varied among the effector cell populations obtained from the different donors. Thus, the cytolytic activity of four different CD3<sup>+</sup>CD8<sup>+</sup> populations against A431

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cells ranged from 45 to 70% of  $^{51}\text{Cr}$  release at a 10:1 effector to target cell ratio.

Ca<sup>++</sup> mobilization assay

The concentration of cytoplasmic free Ca<sup>++</sup> was determined in CD8+ 5 polyclonal cell lines as described previously by Pantaleo et al., 1986, Eur. J. Immunol., 16, 1639-1644. Cells ( $2.5 \times 10^6$ ) were stained with the acetoxymethylester of Fura-2 (1  $\mu\text{M}$  final concentration; Sigma, St. Louis, MO, USA), and the fluorescence of the cellular suspension was monitored with Spectrofluorimeter (Perkin Elmer Corp., Pomona, CA, USA) using a 2 10 ml quartz cuvette. Cell suspensions were excited at 345 nm and fluorescence was measured at 496 nm. All determinations were performed at 37°C using a thermostatically controlled cuvette holder and a stirring apparatus.

Since bimAbs produced by hybrid hybridomas possess only one binding site 15 for each specificity recognized (Milstein and Cuello, 1984, Immunol. Today, 5, 299-304), the authors of the present invention compared the DSM ACC2151 bimAb with the parental antiCD3 mAb for its ability to deliver activation signals to the T cells. In Ca<sup>++</sup> mobilization experiments performed on CD8+ polyclonal cell lines, the DSM ACC2151 bimAb failed to 20 induce intracellular Ca<sup>++</sup> increase (Figure 4, panel C). However, cross-linking of surface CD3 molecules, induced by the subsequent addition of an anti-mouse IgG antibody, resulted in a rapid increase of intracellular Ca<sup>++</sup> levels. At variance from DSM ACC2151 bimAb, the parental bivalent 25 anti-CD3 mAb produced a significant Ca<sup>++</sup> mobilization, even in the absence of the cross-linking reagent, which could be further increased by addition of the anti-mouse Ig antibody (Figure 4, panel B). The present authors next investigated whether T cells coated with the DSM ACC2151 bimAb retained for a prolonged period of time their ability to mobilize

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Ca<sup>++</sup> upon cross-linking of the cell surface bound bimAb molecules. As shown in Figure 4, panel D, T cells pretreated with bimAb and cultured at 37°C up to 24 hours efficiently mobilized Ca<sup>++</sup> in response to anti-mouse Ig addition. In parallel control experiments, anti-mouse Ig antibodies alone failed to activate effector cells (Figure 4, panel A). Moreover, 5 the same DSM ACC2151 bimAb-coated cultured effector cells efficiently lysed EGF-R+ target cells, thus confirming that DSM ACC2151 bimAb-armed effector cells retain their ability to recognize and lyse the relevant tumors, previously described, for a prolonged time period (Figure 5).

10 Lymphokine production assay

Effector cells were stimulated with target cells, with parental antibodies or DSM ACC2151 bimAb in different combination. As a positive control, soluble anti-CD3 mAb plus 2 ng/ml of phorbol 1,2-myristate-1,3-acetate (PMA) were used. Following 18 hours of culture, cell free supernatants were collected and tested for lymphokine presence. GM-CSF, 15 IFN-gamma and TNF- $\alpha$  were measured using the EASIA kits (Enzyme Amplified Sensitivity Immunoassay, Medgenix Diagnostics, Brussels, Belgium) following instructions supplied by the manufacturer. Data were expressed as pg/ml for GM-CSF and TNF- $\alpha$ , whereas IU/ml were used for IFN-gamma.

20 Appropriate dilutions of recombinant lymphokines were always included as standards for each assay.

The present authors also investigated whether DSM ACC2151 bimAb-coated lymphocytes could secrete lymphokines upon interaction with the relevant target cells.

25 Table 3 shows that the secretion of lymphokines in a CD8+ polyclonal cell line is induced by co-stimulation with DSM ACC2151 bimAb and the specific target cells.

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Table 3

	GM-CSF	TNF- $\alpha$	INF-gamma
Controllo	<0 <sup>a</sup>	<0 <sup>a</sup>	<0 <sup>b</sup>
PMA + $\alpha$ CD3	>20000	>20000	200
A431	750	490	30
A431 + $\alpha$ CD3 + $\alpha$ EGF-R	1000	2980	40
A431 + bimAb	>20000	>20000	>240
bimAb	1000	4500	38
NIH-3T3 + bimAb	505	<0	12

<sup>a</sup> = concentration expressed in pg/ml<sup>b</sup> = concentration expressed in UI/ml

As shown in Table 3, anti-CD3 was able to induce secretion of IFN-gamma, TNF- $\alpha$  and GM-CSF in polyclonal T cell lines in the presence of phorbol ester PMA as a co-stimulus. When EGF-R+ tumor cells were added to untreated lymphocytes, only low levels of lymphokine secretion could be achieved, while maximal lymphokine production was induced by the simultaneous use of bimAb and relevant target cells. On the other hand, EGF-R- tumor cell lines and bimAb did not induce significant lymphokine secretion.

Example 3Bispecific antibody purification

Since DSM ACC2151 bimAb represented only a fraction of the antibody molecules secreted by hybrid hybridomas (Milstein and Cuello, 1984, Immunol. Today, 5, 299-304), the present authors purified the DSM ACC2151 bimAb by protein A chromatography followed by cation exchange chromatography. DSM ACC2151 bimAb was purified from hybrid hybridoma culture supernatant. Cells producing bimAb were grown in serum-free medium in Acusyst R hollow fiber bioreactor (Endotronics). Conditioned media were collected and diluted 1:1 in Protein A binding buffer (1.5 M

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glycine pH 8.9; 150 mM NaCl) for processing by a first step of affinity chromatography on a  $\times$ k 26 column of Protein A-Sepharose Fast Flow (Pharmacia). Protein A elution buffer is: 0.1 M sodium citrate pH 3.6. Eluted material from Protein A was analyzed by MonoS chromatography as reported in Figure 6 showing the presence of DSM ACC2151 bimAb distributed in two peaks (1 and 2) and the presence of parental  $\alpha$ CD3 DSM ACC2152 and  $\alpha$ EGF-R DSM ACC2150 mAbs. Thus, eluted material from Protein A was diluted 1:1 in buffer A (25 mM sodium acetate pH 5.0) and located on  $\times$ k 26 column of S-Sepharose FF (Pharmacia) conditioned with buffer A plus 50 mM NaCl. Elution was performed by a step procedure with buffer B made of buffer A plus 1 M NaCl. The steps were 20%, 25% and 30% of buffer B. Fractions from S-Sepharose chromatography were analyzed by MonoS chromatography and fractions corresponding to peak 1 of Figure 6 were pooled and confirmed to be DSM ACC2151 bimAb according to IEF pattern and 10 functional activity. Figure 7 shows the analytical MonoS chromatography of purified DSM ACC2151 bimAb. Purity was further confirmed by SDS-PAGE analysis on Phast gel 4-15% (Pharmacia) (Figure 8).

Figure 8 shows a SDS-PAGE analysis exhibiting the electrophoretic mobility in both reducing and non-reducing conditions. Lanes 1 and 20 contain the purified DSM ACC2151 bimAb. Lane 3 contains parental  $\alpha$ CD3 mAb and lanes 4 and 5 contain  $\alpha$ EGF-R and  $\alpha$ CD3 standard in mAbs. The presence of the highest concentration of bifunctional molecules of bimAb DSM ACC2151 in purified peak 1 was confirmed by cytolytic assay against A<sup>431</sup> target cells.

25 Comparison of the DSM ACC2151 bimAb activity with the Ferrini bimAb activity

DSM ACC2151 bimAb was prepared and purified as above described.

Ferrini bimAb was obtained by Dr. Ferrini (as previously specified).

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The activity of the two anti-EGF-R/anti-CD3 bimAbs was analysed testing the cytolitic activity of a CD8+ cytolitic cell line against A431 target cells in the presence of different concentrations of bimAb. As shown in Figure 9, DSM ACC2151 bimAb was significatively stronger to induce 5 cytolitic activity at low concentration of bimAb if compared to Ferrini bimAb. IC<sub>50</sub> calculated for the two bimAbs (Fig.9) shows that DSM ACC2151 bimAb is about 10 times more active than Ferrini bimAb. According to the evaluation of the activities of the above cited bimAbs, then it results surprising the different behaviour (that maybe could indicate a different 10 mechanism of action) of the two bimAbs studied.

A DNA sequence coding for an antibody according to the invention can be prepared. Said sequence, eventually, can be inserted into a plasmid vector, preferably under the control of a promoter which enables its expression in a desired host cell.

15 Said host cell is selected from the group consisting of bacterial, yeast, insect, plant or mammalian cells.

The present invention is therefore directed also to a method for the production of a monoclonal antibody or fragments thereof according to the invention comprising the cultivation of a host cell under conditions 20 appropriate for the expression of said DNA sequence and recovering the product from the culture.

An antibody according to the present invention is also useful in therapy.

An antibody or its fragments [for example F(ab')<sub>2</sub>] according to the 25 invention is also used for the preparation of a pharmaceutical composition, useful for the treatment of tumors, in combination with a pharmaceutically acceptable carrier and/or excipient.

A pharmaceutical composition according to the present invention therefore

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comprises a monoclonal antibody or its fragments according to the invention for the treatment of tumors, optionally in combination with a pharmaceutically acceptable carrier and/or excipient.

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Claims

- 1 1. A DSM ACC2151 hybrid hybridoma secreting an anti-EGF-R/anti-CD3 bispecific monoclonal antibody of IgG1/IgG2a hybrid isotype.
- 1 2. The anti-EGF-R/anti-CD3 bispecific monoclonal antibody of IgG1/IgG2a hybrid isotype according to claim 1.
- 1 3. A DSM ACC2152 hybridoma secreting an anti-CD3 monoclonal antibody of IgG2a isotype.
- 1 4. The anti-CD3 monoclonal antibody according to claim 3.
- 1 5. The monoclonal antibody according to claim 4 comprising: the sequence of the heavy chain variable region (VH) reported in SEQ ID NO:1; and the sequence of the light chain variable region (LH) reported in SEQ ID NO:3.
- 1 6. A bispecific monoclonal antibody obtainable by the somatic hybridization of the DSM ACC2150 hybridoma secreting an anti-EGF-R monoclonal antibody with a hybridoma secreting an anti-CD3 monoclonal antibody.
- 1 7. A bispecific monoclonal antibody obtainable by the somatic hybridization of a hybridoma secreting an anti-EGF-R monoclonal antibody with the DSM ACC2152 hybridoma secreting an anti-CD3 monoclonal antibody.
- 1 8. A recombinant single chain bispecific monoclonal antibody according to claim 6 obtainable by gene fusion of heavy and light chain variable region sequences of the genes encoding an anti-CD3 monoclonal antibody and an anti-EGF-R monoclonal antibody secreted by DSM ACC2150 hybridoma.
- 1 9. A recombinant single chain bispecific monoclonal antibody according to claim 7 obtainable by gene fusion of heavy and light chain variable region sequences of the genes encoding an anti-CD3 monoclonal antibody secreted by DSM ACC2152 hybridoma and an anti-EGF-R monoclonal antibody.
- 1 10. A recombinant single chain bispecific monoclonal antibody according

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2 to claims 8-9 obtainable by gene fusion of heavy and light chain variable  
3 region sequences of the genes encoding an anti-CD3 monoclonal antibody  
4 secreted by DSM ACC2152 hybridoma and an anti-EGF-R monoclonal antibody  
5 secreted by DSM ACC2150 hybridoma.

1 11. A method for the production of a hybrid hybridoma secreting an anti-  
2 EGF-R/anti-CD3 bispecific monoclonal antibody of IgG1/IgG2a hybrid  
3 isotype comprising the somatic hybridization of the DSM ACC2150 hybridoma  
4 secreting an anti-EGF-R monoclonal antibody and a hybridoma secreting an  
5 anti-CD3 monoclonal antibody.

1 12. A method for the production of a hybrid hybridoma secreting an anti-  
2 EGF-R/anti-CD3 bispecific monoclonal antibody of IgG1/IgG2a hybrid  
3 isotype comprising the somatic hybridization of a hybridoma secreting an  
4 anti-EGF-R monoclonal antibody and the DSM ACC2152 hybridoma secreting an  
5 anti-CD3 monoclonal antibody.

1 13. A method according to claim 11 for the production of an anti-EGF-  
2 R/anti-CD3 bispecific monoclonal antibody comprising the somatic  
3 hybridization of the DSM ACC2150 hybridoma and a hybridoma secreting an  
4 anti-CD3 monoclonal antibody and the following purification of said  
5 antibody by a protein-A chromatography and then by a cation exchange  
6 chromatography.

1 14. A method according to claim 14 for the production of an anti-EGF-  
2 R/anti-CD3 bispecific monoclonal antibody comprising the somatic  
3 hybridization of a hybridoma secreting an anti-EGF-R monoclonal antibody  
4 and the DSM ACC2152 hybridoma and the following purification of said  
5 antibody by a protein-A chromatography and then by a cation exchange  
6 chromatography.

1 15. A method according to claims 13-14 for the production of an anti-EGF-  
2 R/anti-CD3 bispecific monoclonal antibody comprising the somatic

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3 hybridization of the DSM ACC2150 hybridoma and the DSM ACC2152 hybridoma  
4 and the following purification of said antibody by a protein-A  
5 chromatography and then by a cation exchange chromatography.

1 16. A DNA sequence encoding the antibody or its fragments according to  
2 claim 2, 6-7.

1 17. A recombinant DNA molecule comprising the DNA sequence or its  
2 portions according to claim 16.

1 18. The recombinant DNA molecule of claim 17 wherein said DNA sequence is  
2 under the control of a promoter which enables its expression in a desired  
3 host cell.

1 19. A host cell containing a recombinant DNA molecule according to claims  
2 17-18.

1 20. The host cell of claim 19 wherein said cell is selected from the  
2 group consisting of bacterial, yeast, insect, plant and mammalian cells.

1 21. A method for the production of a monoclonal antibody or fragments  
2 thereof comprising the cultivation of a host cell according to claim 20  
3 under conditions appropriate for the expression of the DNA sequence  
4 according to claims 17-18 and recovering the product from the culture.

1 22. An antibody according to claims 2, 6-7 for use in therapy.

1 23. A pharmaceutical composition comprising a monoclonal antibody or its  
2 fragments according to claims 2, 6-7 for the treatment of tumors,  
3 optionally in combination with a pharmaceutically acceptable carrier  
4 and/or excipient.

1 24. Use of an antibody according to claims 2, 6-7 for the preparation of a  
2 pharmaceutical composition, useful for the treatment of tumors, in  
3 combination with a pharmaceutically acceptable carrier and/or excipient.

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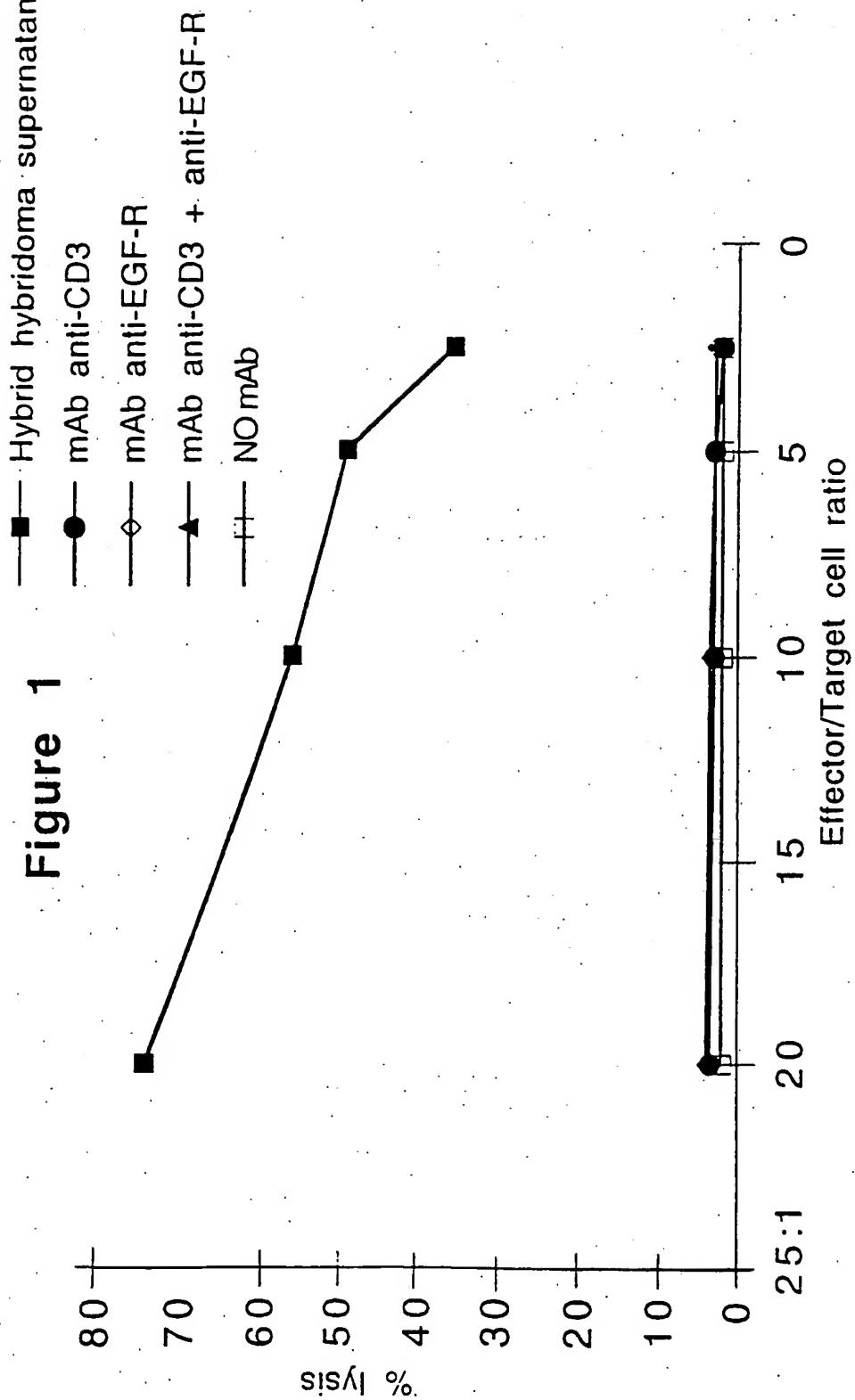
Anti-EGF-R/anti-CD3 bispecific monoclonal antibody, method for its production and its use.

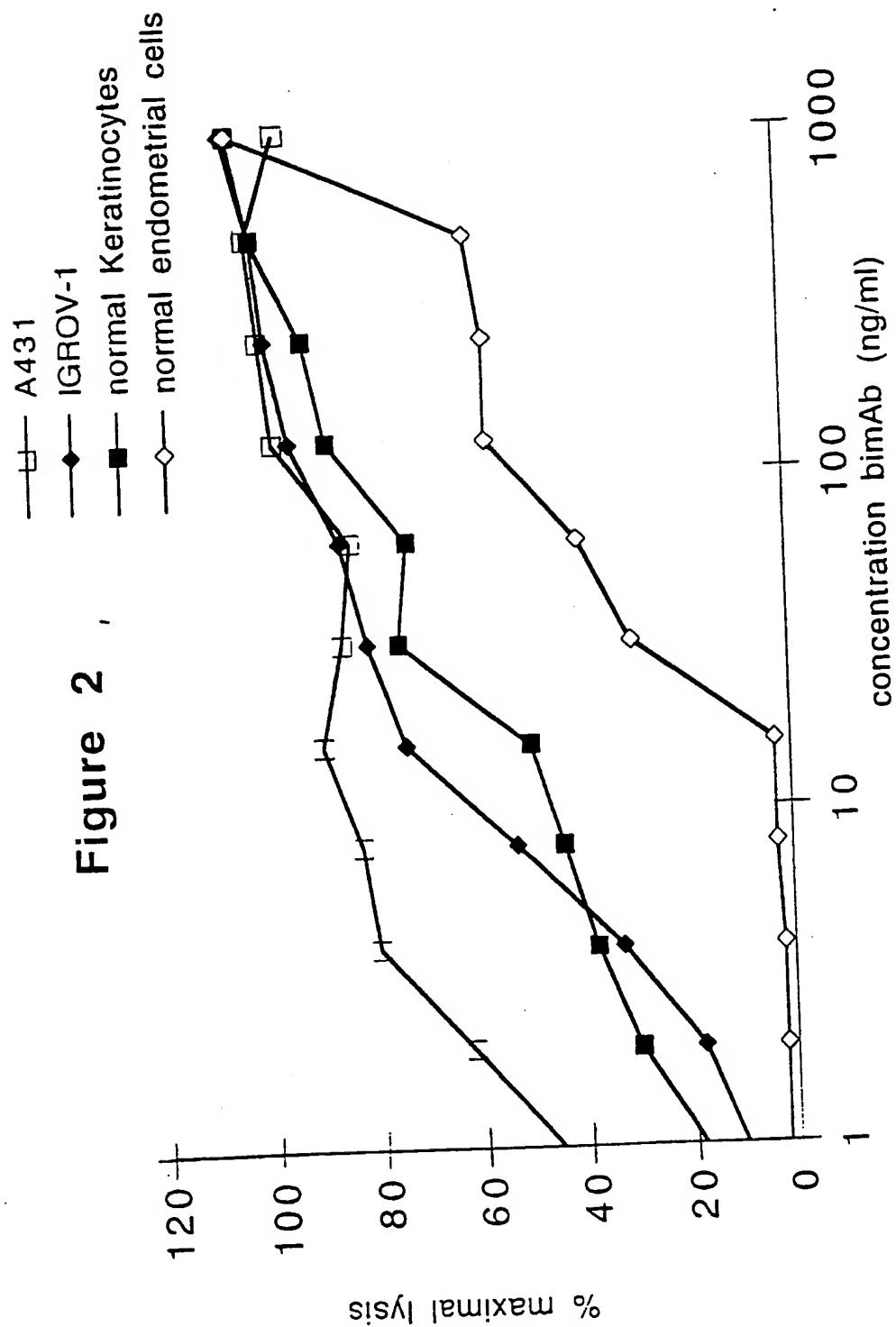
Abstract

It is described an anti-EGF-R/anti-CD3 bispecific monoclonal antibody 5 (bimAb) of hybrid isotype (IgG1/IgG2a). It is also described the construction of a hybrid hybridoma secreting such bimAb, and the purification of anti-EGF-R/anti-CD3 bimAb molecules from hybrid hybridoma performed by protein-A cation exchange chromatography. Said bimAb turned out to be useful against tumor cells showing the epidermal growth factor 10 receptor (EGF-R+).

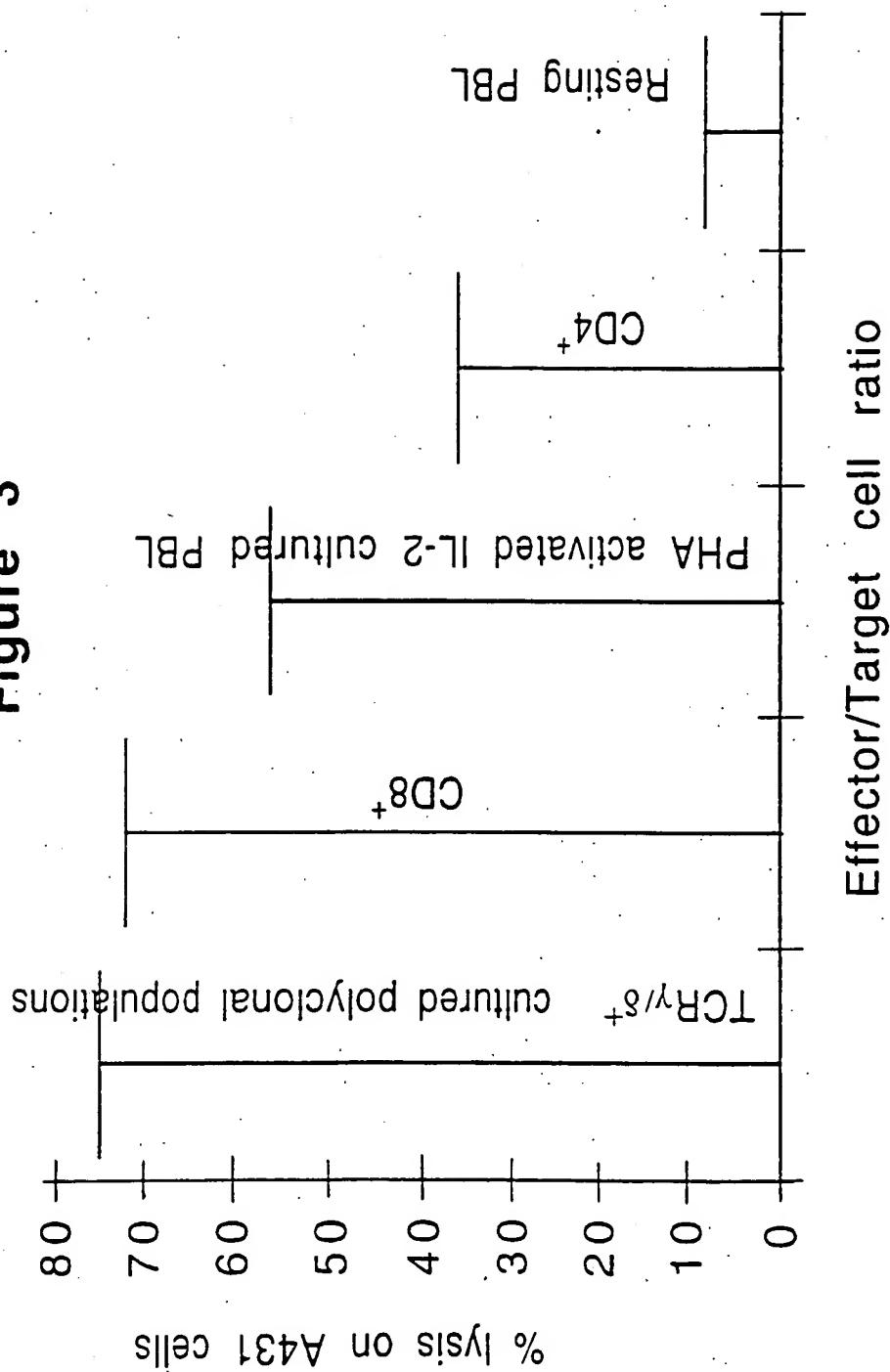
1/9

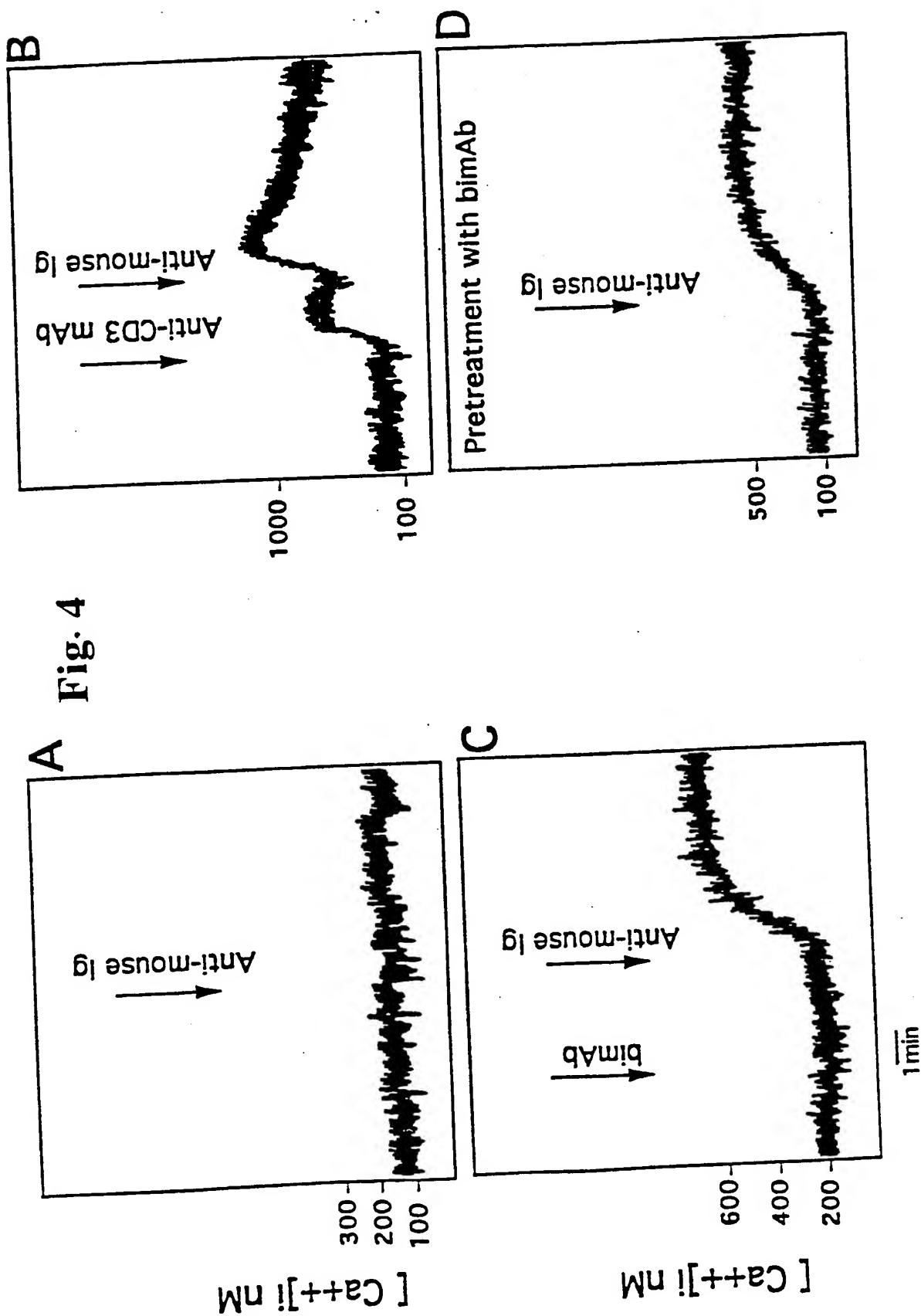
Cytolytic activity in the presence of:





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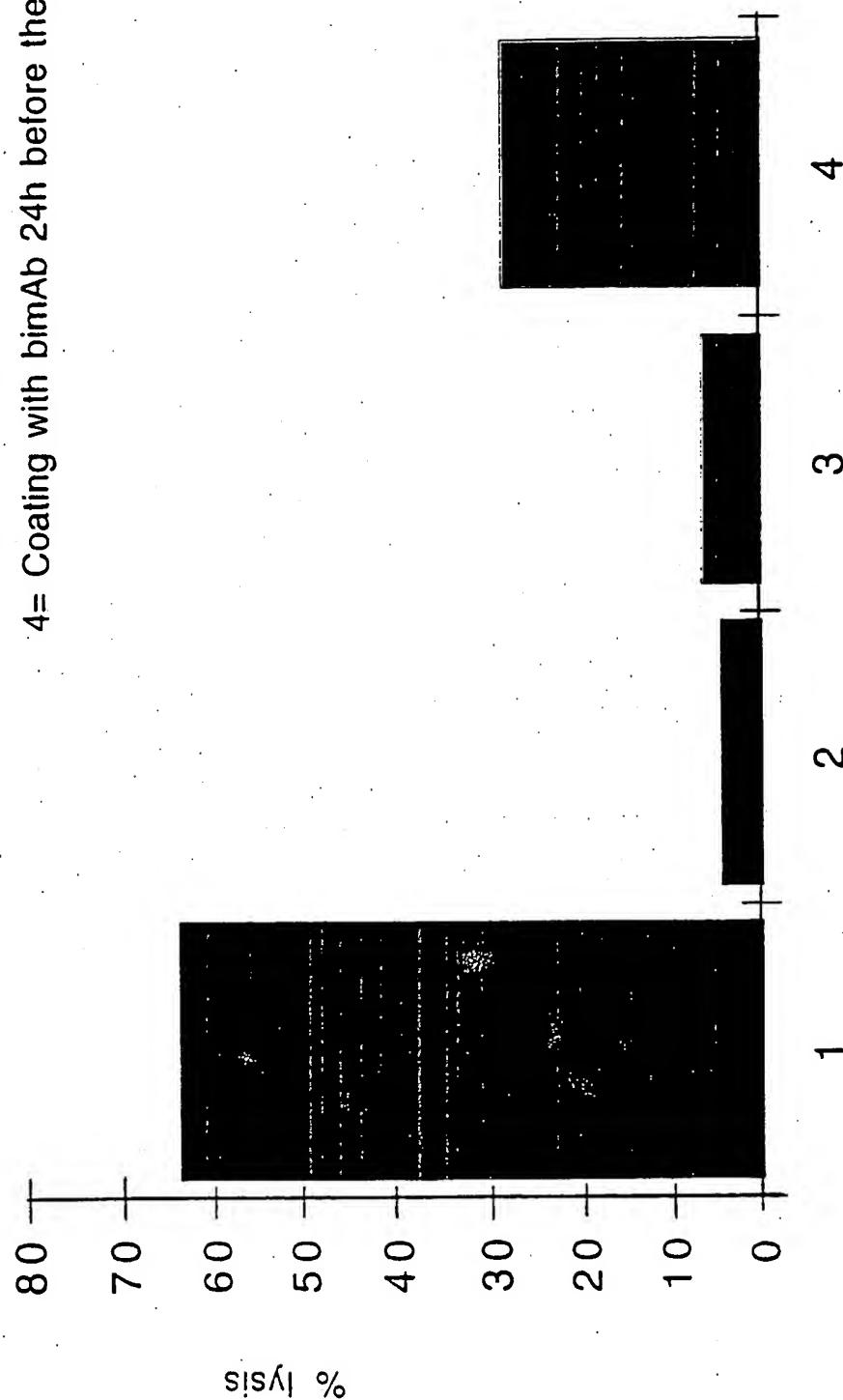
**Figure 3**



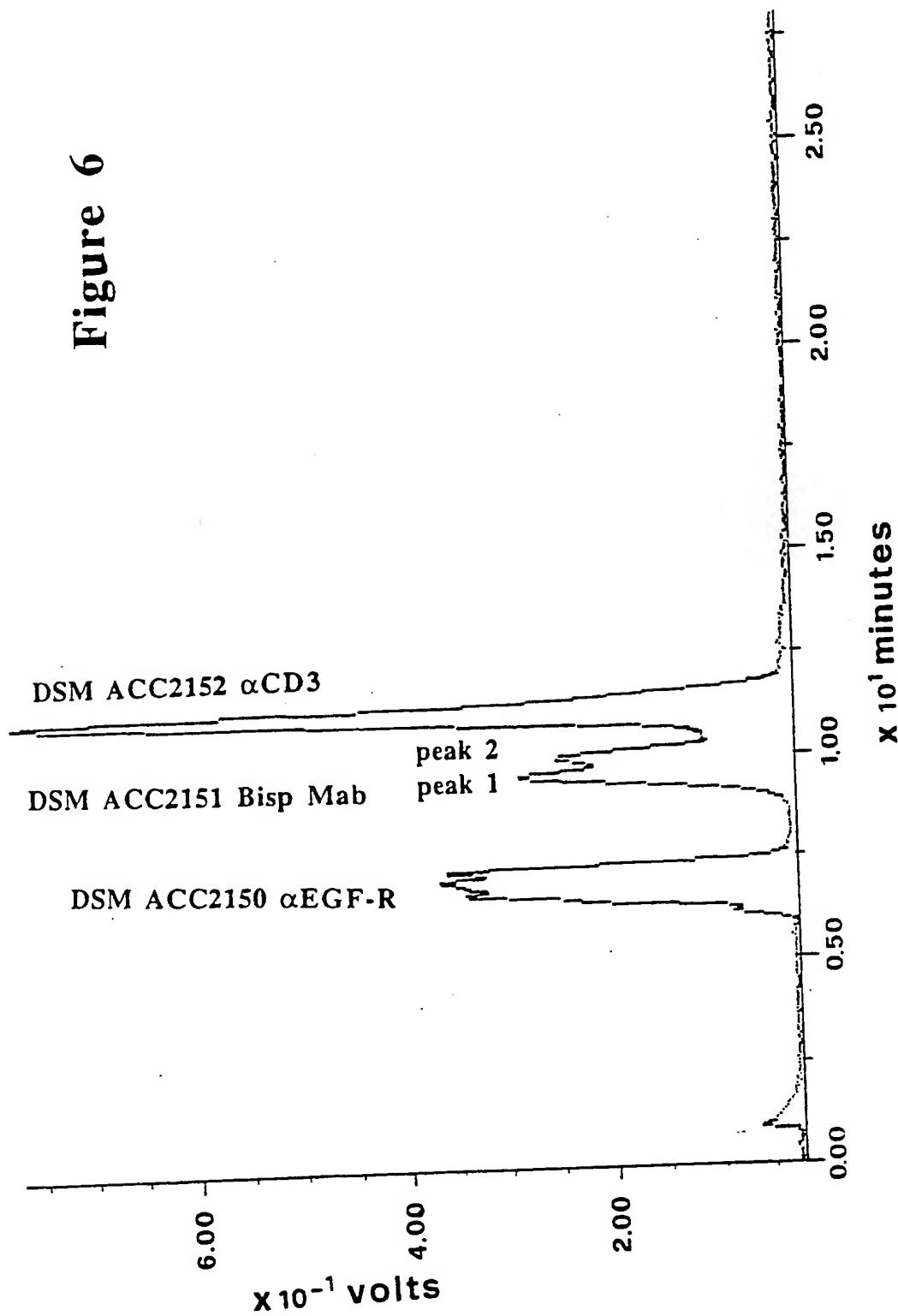
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- 1= bimAb added to the test  
2= NO Ab  
3= mAb anti CD3 + anti-EGF-R  
4= Coating with bimAb 24h before the test

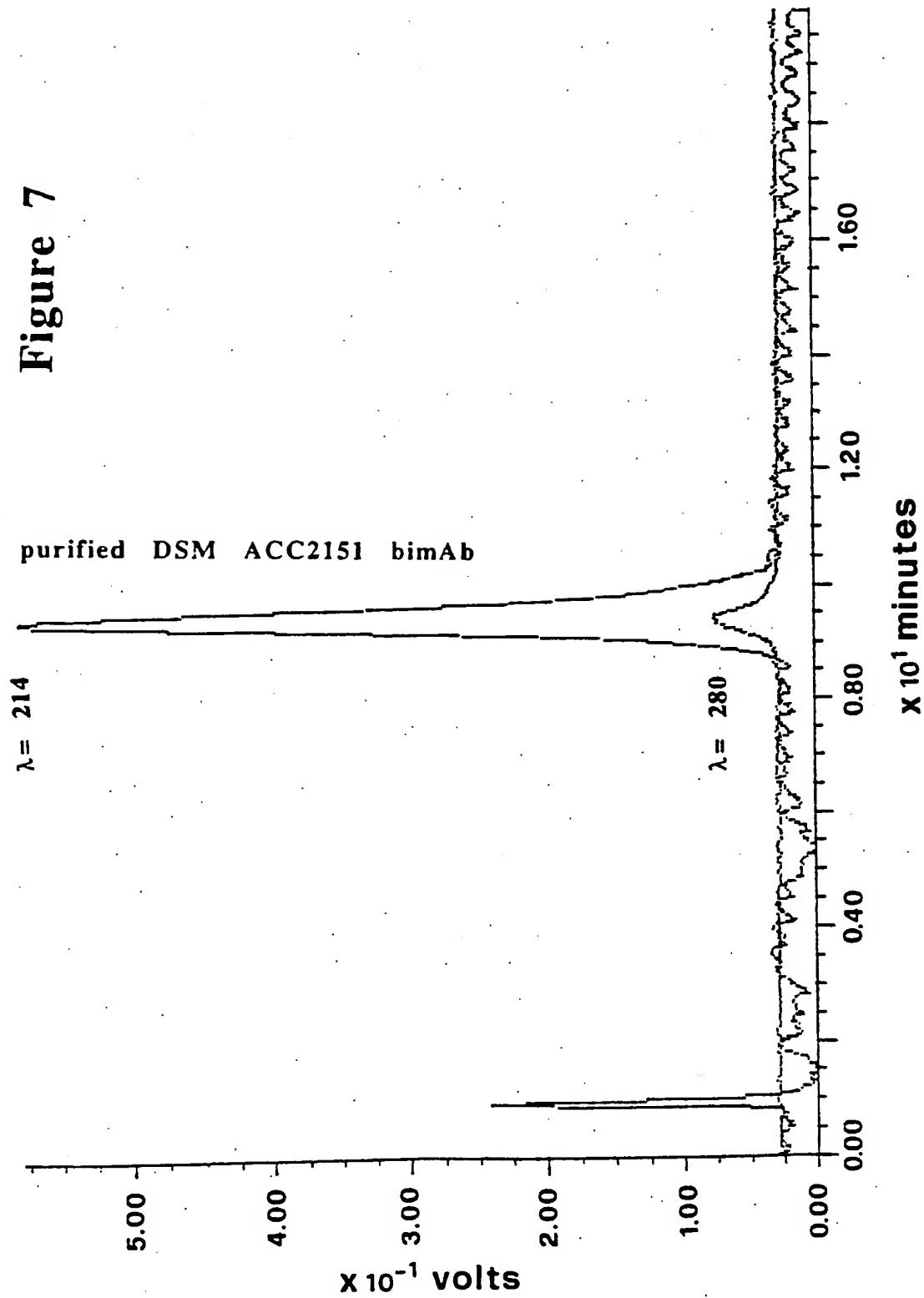
**Figure 5**



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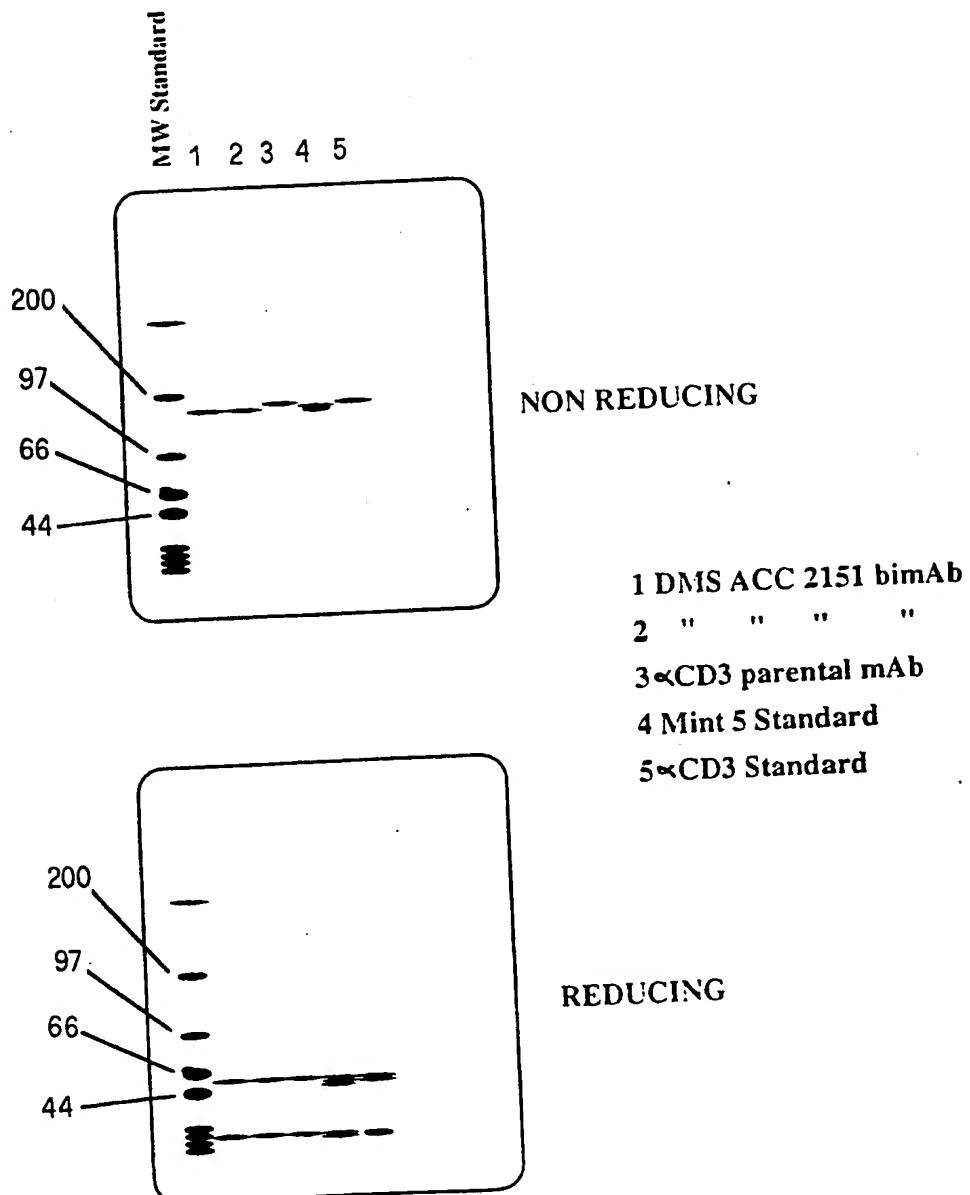
**Figure 6**

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**Figure 7**

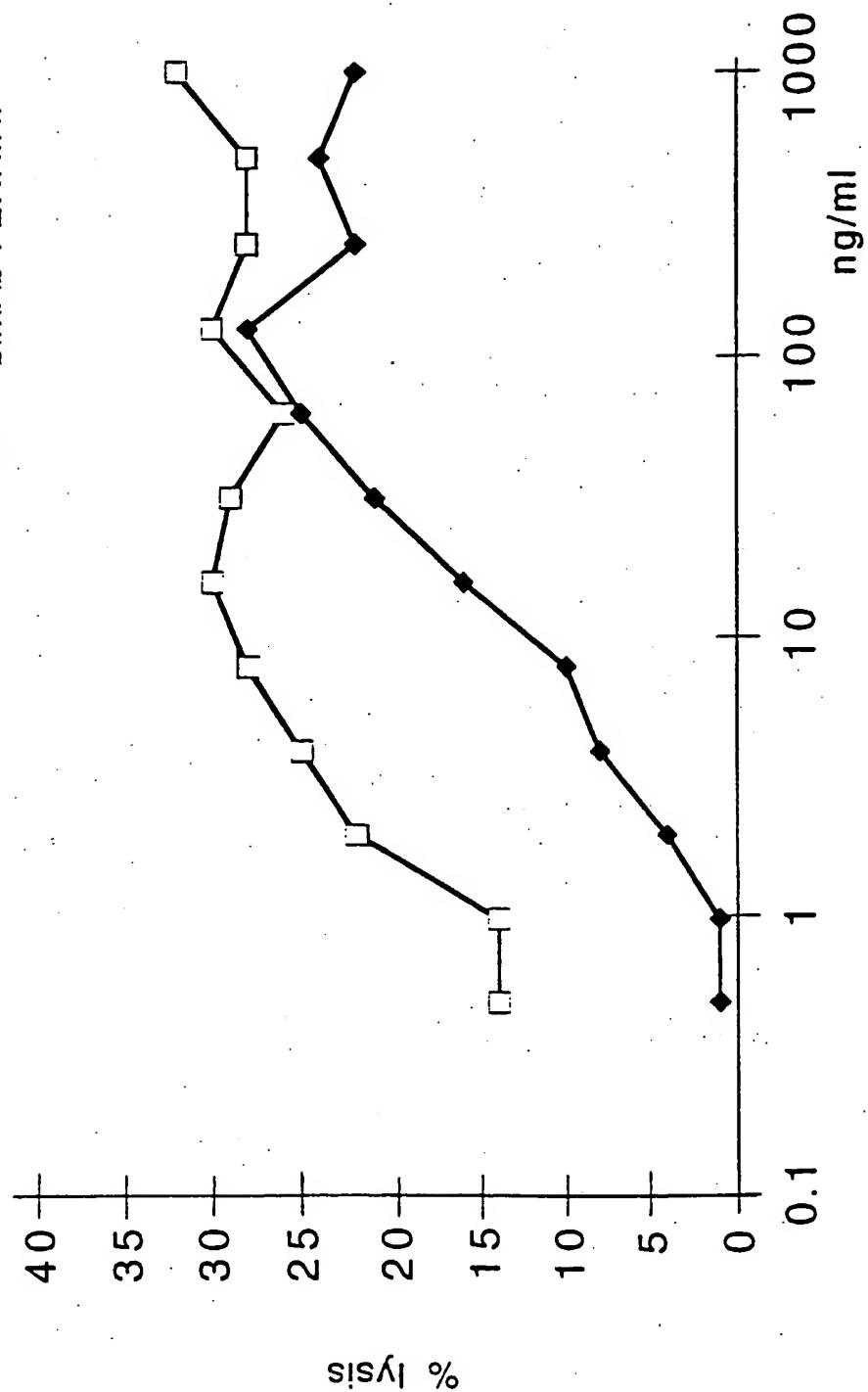
# Figure 8

Phast gel 4-15 %



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**Figure 9**  
bimAb DSM ACC2151  
bimAb FERRINI



# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 94/03995

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC 6	C12N15/13	C12N5/20	C07K16/46	C07K16/28	C12N15/06
	C12N5/06	A61K39/395			

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>CANCER DETECTION AND PREVENTION, vol.17, no.2, 1993, NEW YORK NY, USA pages 295 - 300</p> <p>S. FERRINI ET AL. 'Use of anti-CD3 and anti-CD16 bispecific monoclonal antibodies for the targeting of T and NK cells against tumor cells.' cited in the application see the whole document ----</p> <p style="text-align: right;">-/-</p>	1-7, 11-24

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

\* Special categories of cited documents :

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- "E" earlier document but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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1

Date of the actual completion of the international search

Date of mailing of the international search report

10 March 1995

20 -03- 1995

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## INTERNATIONAL SEARCH REPORT

Internati Application No  
PCT/EP 94/03995

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	INTERNATIONAL JOURNAL OF CANCER, SUPPLEMENT, vol.7, 1992, GENEVA, SWITZERLAND pages 15 - 18 S. FERRINI ET AL. 'Targeting of T or NK lymphocytes against tumor cells by bispecific monoclonal antibodies: Role of different triggering molecules.' see the whole document ---	1-7, 11-24
X	PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH, vol.34, March 1993, USA page 476 S. FERRINI ET AL. 'Targeting of T lymphocytes by anti-CD3/anti-EGF-R bispecific monoclonal antibodies. Role of adhesion molecules.' see abstract 2840 ---	1-7, 11-24
X	THE FASEB JOURNAL, vol.6, no.5, 28 February 1992, BETHESDA MD, USA page A2059 D. MEZZANZANICA ET AL. 'Use of bispecific mAbs, anti-CD3/anti-EGF-R, to retarget human activated T lymphocytes against tumor cells over-expressing EGF-R.' see abstract 6506 ---	1-7, 11-24
A	THE JOURNAL OF IMMUNOLOGY, vol.146, no.3, 1 February 1991, BALTIMORE MD, USA pages 1067 - 1071 S. GILLIES ET AL. 'Targeting human cytotoxic T lymphocytes to kill heterologous epidermal growth factor receptor-bearing tumor cells.' see the whole document ---	8-10
A	THE JOURNAL OF EXPERIMENTAL MEDICINE, vol.175, January 1992, NEW YORK NY, USA pages 217 - 225 M. SHALABY ET AL. 'Development of humanized bispecific antibodies reactive with cytotoxic lymphocytes and tumor cells overexpressing the HER2 protooncogene.' see the whole document ---	1-24
		-/-

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 94/03995

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		Relevant to claim No.
Category	Citation of document, with indication, where appropriate, of the relevant passages	
A	<p>EUROPEAN JOURNAL OF CANCER, vol.27, no.SUP3, 1991, OXFORD, GB page 557 E. TOSI ET AL. 'The anti-EGFR MINT5 mAb is able to specifically target RIP alpha-sarcin cytotoxicity against relevant target cells.' see abstract 7.041 -----</p>	1-24
P,X	<p>INTERNATIONAL JOURNAL OF CANCER, vol.55, no.6, 2 December 1993, GENEVA, SWITZERLAND pages 931 - 937 S. FERRINI ET AL. 'Targeting of T lymphocytes against EGF-receptor+ tumor cells by bispecific monoclonal antibodies: Requirement of CD3 molecule cross-linking for T cell activation.' see the whole document -----</p>	1-7, 11-24

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